TOXIC TERPENOIDS FROM HIGHER FUNGI AND THEIR POSSIBLE ROLE IN CHEMICAL DEFENCE SYSTEMS

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ABSTRACT - A considerable number of biologically active terpenoids, possessing for example antibiotic, antifeedant and mutagenic activities, have been isolated from mushrooms. For most of them the biological activity is caused by the presence of a reactive chemical functionality (e.g. a α,β-unsaturated ketone or lactone), and it has been proposed that many of them in fact are defensive compounds. The cytotoxic sesquiterpenes responsible for the strong taste of the fruit bodies of pungent *Lactarius* species are good examples. These sesquiterpenes are not present in the intact fruit bodies, but are formed enzymatically from a biologically inactive and tasteless sesquiterpenoid precursor as a response to injury. Different species, e.g. *L. vellereus*, *L. piperatus* and *L. necator*, form different combinations of pungent sesquiterpenes, and it is possible to distinguish between very close species by comparing the sesquiterpenes formed ■ a response to injury.

RÉSUMÉ - Un nombre considérable de terpénoïdes biologiquement actifs, ayant par exemple des activités antibiotiques, répulsives et mutagènes, ont été trouvés dans des champignons. Pour un grand nombre d'entre eux, les activités biologiques sont liées à la présence de fonctions chimiques réactives (p. ex. cétones et lactones α,β-insaturées), et il a été proposé qu'ils étaient en réalité des composés de défense. Les sesquiterpènes cytotoxiques responsables du goût âcre de la chair d'espèces de *Lactarius* piquantes en sont un exemple. ▮ est intéressant de noter que les sesquiterpènes ne sont pas présents originairement dans la chair du champignon, mais sont ultérieurement formés de façon enzymatique à partir de précurseurs sesquiterpénoïdes biologiquement inactifs et n'ayant aucun goût lorsque le champignon est attaqué. Différentes espèces comme *L. vellereus*, *L. piperatus* ■ *L. necator*, forment différents ensembles de sesquiterpènes piquants en tant que réponse à une attaque, et il est possible de distinguer des espèces très proches par leurs sesquiterpènes formés.

INTRODUCTION

The number of terpenoids isolated from Nature is large, more than 22,000 compounds have been reported in the literature (Connolly & Hill, 1991), and the kingdom of Fungi have proven to be a rich source. Although only few of the classical mushroom poisons belong to this class of natural products, many fungal terpenoids possess potent biological activities and are toxic to man (Bresinsky & Besl, 1985; Anke & Steglich, 1988). A few examples are shown in Figure 1.

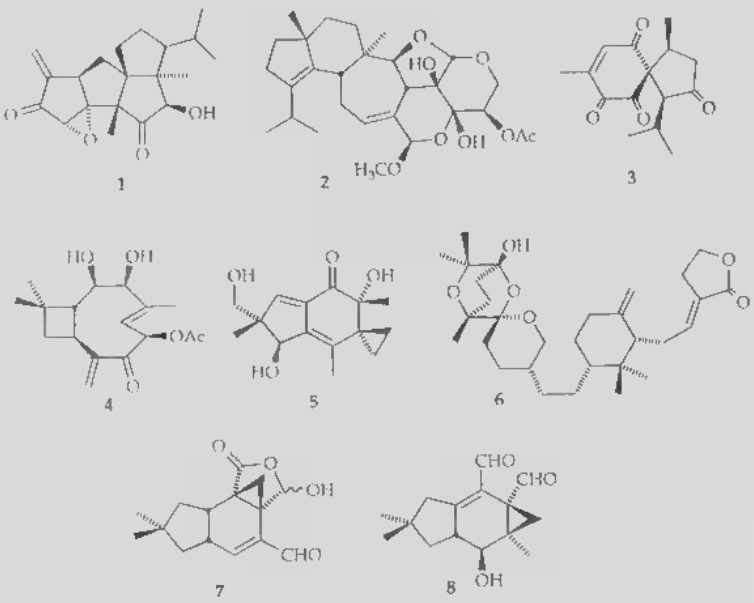

Figure 1

The crinipellins (e.g. crinipellin A [**1**]), are cytotoxic diterpenoids isolated from cultures of *Crinipellis stipitaria* (Anke *et al.*, 1985), while the striatins (e.g. striatin A [**2**]) are produced by both cultures (Hecht *et al.*, 1978) and fruit bodies (Rabe, 1989) of *Cyathus striatus* as well as by other species. The LD_{50} value for striatin A (**2**) is 60-110 mg/kg (i.p. administration to tumour-bearing mice) (Douros & Anke, 1994). The acorane hemimycin (**3**) was isolated from cultures of *Hemimycena cucullata* and *H. candida*, and possess potent cytotoxic activity (Bäuerle *et al.*, 1986). The fruit bodies of *Hypholoma fasciculare* have yielded a series of toxic triterpenoids, the fasciculols (Suzuki *et al.*, 1983), and from cultures of several *Hypholoma* species the cytotoxic caryophyllane naematolon (**4**) was obtained (Backens *et al.*, 1984). However, the toxicity of the latter toward mammals appear to be limited, as the LD_{50} value is well above 225 mg/kg (i.p. administration to tumour-bearing mice) (Douros & Anke, 1994). Fruit bodies and cultures of *Omphalotus olearius* produce the toxic but also antineoplastic illudane illudin S (**5**) (McMorris & Anchel, 1963). Several cytotoxic triterpenes, the saponaceolides, have been isolated from *Tricholoma* species, e.g. *T. saponaceum* (De Bernardi *et al.*, 1988; 1991), and although their cytotoxic activity is remarkable (for saponaceolide B (**6**) ID_{50} on the LoVo cell line is 0.16 µg/ml, and LD_{50} on brine shrimps is 40 ng/ml) they possess no antimicrobial activity (De Bernardi *et al.*, 1991). Two further examples of toxic sesquiterpenes are the mutagenic (Anke & Sterner, 1991) marasmic acid (**7**) and merulidial (**8**), originally isolated from *Marasmius conigenus* (Kavanagh *et al.*, 1949) and *Merulius tremellosus* (Giannetti *et*

al., 1986), respectively. The LD_{50} value for marasmic acid (**7**) is 15-30 mg/kg (i.p. administration to tumour-bearing mice) (Douros & Anke, 1994).

THE SESQUITERPENES OF THE RUSSULACEAE SPECIES

In general, nothing is known about any possible function of the terpenoids in the fungi. However, in the fruit bodies of the pungent species belonging to the genus *Lactarius* (family Russulaceae of the Basidomycotina subdivision) biologically active terpenoids are formed as a response to physical injury, in what appears to be a chemical defence system that protects the fruit bodies against parasites and infections (Camazine *et al.*, 1983; Sterner *et al.*, 1985a). In this, pungent metabolites with antifeedant and antimicrobial activity are formed when an inactive precursor is brought in contact with enzymatic systems by the injury, not unlike a binary weapon system. The precursor is present as an emulsion in the latex of the fruit bodies, this latex is characteristic for the *Lactarius* species and can be observed if a fruit body is cut or broken. The colour and taste of the latex, as well of the flesh, vary between different species, such characters are important taxonomic markers for mycologists and the chemistry related to these differences has been clarified for several species. Interestingly, there seems to be a general pattern within the *Lactarius* genus, also in the non-pungent species, in that the metabolites responsible for the characteristic differences in taste and colour are formed enzymatically from fatty acid ester precursors as a response to injury to the fruit bodies. Depending upon the precursor originally present in the fruit bodies, the *Lactarius* species and the metabolites may be divided into three major groups:

1. The largest is made up by species belonging to the Albati and Lactarius sections, for example *L. vellereus*, *L. piperatus* and *L. scrobiculatus*. They generally have white latex containing large amounts of a biologically inactive fatty acid ester of a marasmane sesquiterpene which rapidly (in seconds) is converted enzymatically to bioactive marasmane, lactarane and seco-lactarane sesquiterpenes as a response to injury (*vide infra*).

2. In the species belonging to the Dapetes section of *Lactarius* (e.g. *L. deliciosus*, *L. deterrimus*, and *L. sanguifluus*) the latex is initially carrot-coloured or wine-coloured but slowly turns green. This has been shown to be due to the presence of stearic acid esters of guaiane sesquiterpenes in the intact fruit bodies which are converted mainly by ester hydrolysis and oxidation to guaiane alcohols and aldehydes in the injured fruit bodies (*vide infra*).

3. The latex and flesh of the fruit bodies of species belonging to the Plinthogali section (e.g. *L. fuliginosus* and *L. picinus*) is originally white and sweet, but due to the enzymatic hydrolysis of the stearic acid ester of a phenolic derivative to the free phenol, which is a potent fungicid, and the enzymatic oxidation of this phenol to a number of derivatives as a response to injury, the latex and flesh turn reddish and bitterly acrid (De Bernardi et al. 1992).

In addition, an isolactarane sesquiterpene has in some investigations been isolated from extracts of *L. rufus* and *L. vellereus* (Daniewski *et al.*, 1976; 1988), species that mainly make marasmane and lactarane sesquiterpenes (*vide infra*). Also, from fruit bodies of species belonging to other sections of *Lactarius*, sesquiterpenes with caryophyllane (isolated from *L. camphoratus* [Daniewski *et al.*, 1981]), drimane (isolated from *L. uvidus* [De Bernardi *et al.*, 1980; 1983]) and glutinopallal (isolated from fruit bodies of *L. glutinopallens* [Fabre-Bonvin & Gluchoff-Fiasson, 1988]) skeletons have also been identified.

A CHEMICAL DEFENCE SYSTEM IN THE PUNGENT *LACTARIUS* SPECIES

In the fruit bodies of *L. vellereus*, the two sesquiterpenes isovelleral (**10**) and velleral (**11**) are formed in seconds from the inactive precursor stearoylvelutinal (**9**) as a response to injury. Isovelleral (**10**) and velleral (**11**) both contain an unsaturated dialdehyde functionality and possess several striking biological activities that suggest that at least part of their function in the injured fruit body should be to discourage parasites and/or to avoid infections (Sterner *et al.*, 1985a; Anke & Sterner, 1991). Large amounts of the two dialdehydes are formed, together the two compounds constitute 6 % of a hexane extract made 10 seconds after injury by grinding a fruit body in a meat grinder (Sterner *et al.*, 1985a). The dialdehydes are subsequently reduced to isovellerol (**12**) and vellerol (**13**), and the reduced derivatives have lost the pungency and most of the biological activities of the unsaturated dialdehydes (Sterner *et al.*, 1985b; 1987).

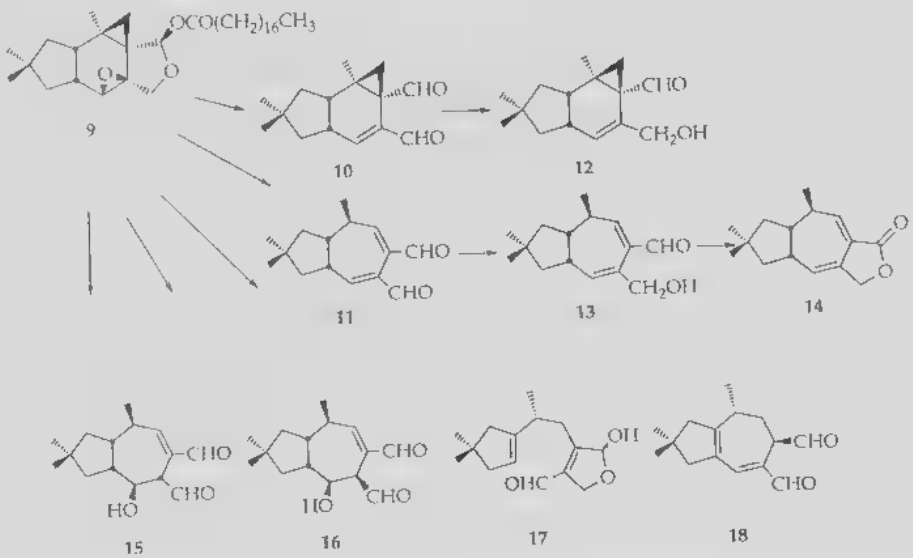

Figure 2

In injured fruit bodies of *L. bertillonii*, only velleral (**11**) is formed from the precursor stearoylvelutinal (**9**), velleral (**11**) is then reduced to vellerol (**13**) which subsequently is oxidised to vellerolactone (**14**) (Hansson *et al.*, 1994), a metabolite not observed in the closely related species *L. vellereus*. Piperdial (**15**) is formed in injured fruit bodies of for example *L. torminosus* (Sterner *et al.* 1985b), while the epimer epi-piperdial (**16**) was isolated from the fruit bodies of *L. necator* (Sterner, 1989). Lactardial (**17**) [containing an unsaturated dialdehyde functionality in disguise and possessing relative modest bioactivities (Anke & Sterner, 1991)] has been isolated from several species (Sterner *et al.*, 1985b), for example *L. necator*, and its status as a natural product is somewhat questionable as it under certain circumstances may be formed as an artefact by chemical transformation of stearoylvelutinal (**9**) (*vide infra*). Chrysorrhedial (**18**) is formed in injured fruit bodies of *L. scrobiculatus* (Pang *et al.*, 1992; De Bernardi *et al.*, 1993) and *L. chrysorrheus* (De Bernardi *et al.*, 1993). The unsaturated dialdehydes **15-18** are by the injured mushroom tissue reduced to the corresponding monoaldehydes, which subsequently are oxidised to the lactones, as velleral (**11**) is reduced to vellerol (**13**) which is oxidised to vellerolactone (**14**).

Besides the potent antimicrobial, cytotoxic and, in some cases, mutagenic activities of the unsaturated dialdehydes isolated from *Lactarius* fruit bodies (Anke & Sterner, 1991), features that are shared with unsaturated dialdehydes isolated from other natural sources such as insects, molluscs and plants, an additional important quality is their intense pungent taste which actually make them excellent as human antifeedants. It has also been shown that mammals that normally feed on mushrooms will avoid edible specimens that have been treated with isovelleral (**10**) (Camazine et al. 1983). However, their pungency probably limits the hazard to consumers of wild mushrooms, as it would be difficult to consume significant amounts of the dialdehydes. 1 μg of for example isovelleral (**10**) (adsorbed on a filter paper disc) distinctly stimulates the taste-buds of the human tongue, and as 1 g of freshly injured *L. vellereus* tissue may contain more than 1000 μg isovelleral (**10**) and velleral (**11**), only small amounts of the raw mushrooms can in practice be consumed by a normal individual. No toxicity tests with mammals have been performed, but the *in vitro* data suggest that the unsaturated dialdehydes would be highly toxic to mammals. The lactones, which appear to be the end-products in several species, also possess cytotoxic activity (De Bernardi *et al.*, 1993), some of them have been reported to possess antifeedant activity against storage pests (Daniewski *et al.*, 1992).

The precursor of all the sesquiterpenes isolated from fruit bodies of the pungent *Lactarius* species is velutinal, present in the intact fruit body as a fatty acid ester [e.g. stearoylvelutinal (**9**)] (Favre-Bonvin *et al.*, 1982; Sterner *et al.*, 1983) as an emulsion (the latex) in specialised hyphae (Gluchoff-Fiasson & Kühner, 1982). Injury apparently brings the precursor in contact with enzymatic systems, and the conversions of stearoylvelutinal (**9**) to isovelleral (**10**), velleral (**11**), piperdial (**15**) and epi-piperdial (**16**) are depicted in Figure 3 (Hansson *et al.* 1991; 1993). Enzymatic β-elimination of the epoxide function of stearoylvelutinal (**9**) formes a metabolite that spontaneously is

transformed to isovelleral (**10**), while the lactaranes (with a seven-membered ring) are believed to be formed via a slightly different mechanism.

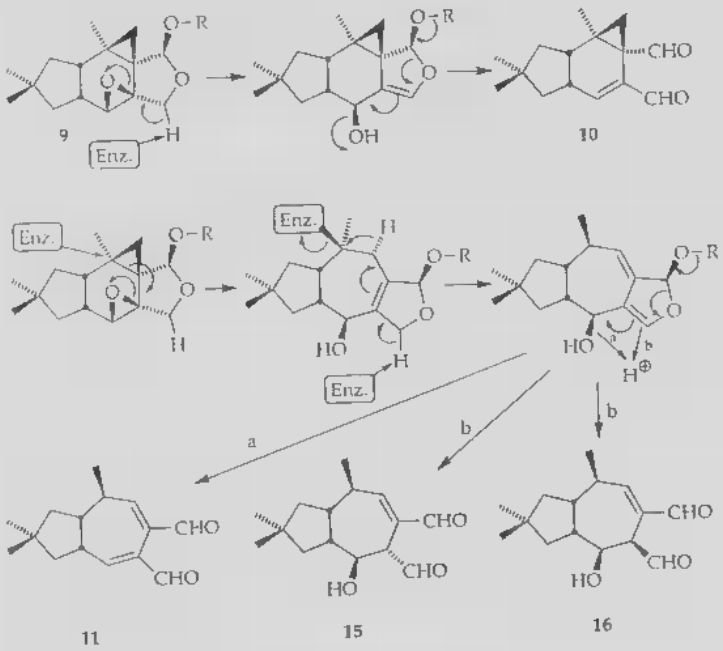

Figure 3

In addition, quite a number of lactarane furans have been reported from the pungent *Lactarius* species, although the majority of the furans are believed to be artefacts formed by chemical transformations of the labile velutinal esters (Sterner *et al.*, 1985c). Traces of acid (present in for instance undistilled solvent or in chromatography gels) will rapidly transform any velutinal derivative to a number of dihydro-hydroxy-(acyloxy)-furans that easily eliminate water to form furans (see Figure 4). However, some furans are co-formed with the dialdehydes, and should be considered as true natural products (Sterner *et al.*, 1988). In addition, it has been observed that the dialdehydes are very sensitive to autoxidation, whereby isovelleral for example is hydroxylated and forms a nor-lactarane via a nor-marasmane (Jonassohn *et al.*, 1995). Many of the Lactarius sesquiterpenes reported have been obtained from extracts of the fruit bodies which have been prepared by soaking the mushrooms in ethanol for several weeks. This was for instance the case for two nor-marasmanes isolated in small amounts from *L. vellereus* (Daniewski *et al.*, 1988), and it is possible that autoxidation of the natural products during extraction and isolation is the origin of some minor metabolites reported as natural products.

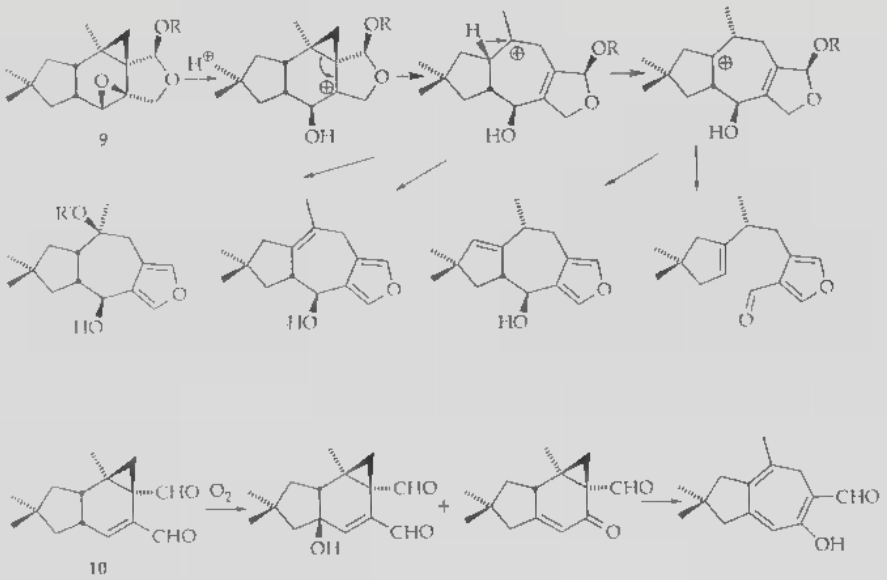

Figure 4

CONVERSIONS OF SESQUITERPENOIDS IN THE SAFFRON MILK CAPS (SECTION DAPETES)

Contrary to the pungent *Lactarius* fruit bodies which only are consumed in exceptional cases, the saffron milk caps are considered to be among the most desirable by consumers of wild mushrooms. They are characterised by strong and with time changing colours of the latex as well as by an agreeable peppery taste, but also by their lack of resistance to parasites compared to the pungent species. The colours of the latex are caused by the presence and formation of azulene and hydroazulene sesquiterpenoids with a guaiane skeleton, and the enzymatic conversions that take place as a response to injury resembles those of the pungent species in that the intact fruit bodies contain fatty acid (mainly stearic acid) esters of sesquiterpenes (esters **19**, **20** and **21**) which are converted to sesquiterpenoic alcohols (compounds **22**, **23** and **24**), aldehydes (compounds **25**, **26** and **27**) and a hydrocarbon (compound **28**). The major differences are that the amounts of sesquitepenoids in the saffron milk caps are much smaller, that the enzymatic conversions are less rapid, and that the chemical functionalities present in the guaiane sesquiterpenes make them considerably less biologically active. In intact fruit bodies of *L. deliciosus* and *L. deterrimus*, only the orange ester **19** could be detected (together with minor amounts of the corresponding linolic acid ester) (Vokac *et al.*, 1970; Bergendorff & Sterner, 1988). As a response to injury, **19** is slowly (minutes) converted by ester hydrolysis and oxidations to the dihydroazulenes alcohol **22** and delicial (**25**), as well as the azulenes deterrol (**24**) and lactaroviolin (**27**)

(Bergendorff & Sterner, 1988). The reduced azulene lactarazulene (**28**) was also detected in extracts of injured specimens, and it is believed that all these sesquiterpenes are formed enzymatically as they never could be observed as transformation products during work-up and isolation. Several of the compounds have been isolated in previous investigations of *L. deliciosus* and *L. deterrimus*, i.e. alcohol **22** (Vokac *et al.*, 1970), lactaroviolin (**27**) (Heilbronner & Schmid, 1953), and lactarazulene (**28**) (Sorm *et al.*, 1954). The green colour of the injured mushroom tissue emerges from a mixture of orange-yellow (**19**, **22** and **25**) and violet-blue (**24** and **27**) compounds. The fruit bodies of *L. sanguifluus* originally contain a mixture of the orange **19** and the red **20**, predominantly the stearic acid esters, which gives the latex its deep red colour (Sterner *et al.*, 1989). Fruit bodies that had been injured (by grinding them in a meat grinder) for 30 minutes prior to extraction, yielded only sangol (**23**) (Sterner *et al.*, 1989), although the aldehyde **26** previously has been isolated from *L. sanguifluus* (De Rosa & De Stefano, 1987). The blue ester **21** has only been isolated from fruit bodies of *L. indigo*, which also yielded lactaroviolin (**27**) (Harmon *et al.*, 1980).

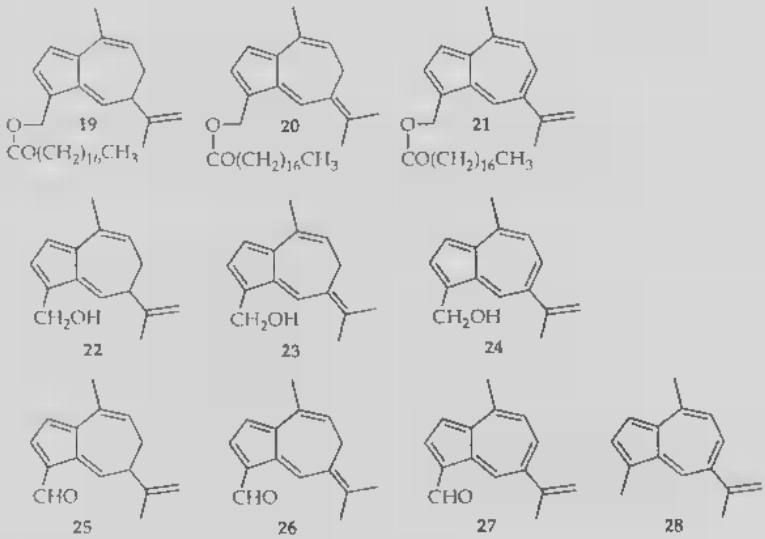

Figure 5

Although the bacteriostatic activity of lactaroviolin (**27**) was reported already in the 1940-ies (Willstaedt & Zetterberg, 1946), the guaiane sesquiterpenoids formed in the saffron milk caps do not appear to affect humans otherwise than by colouring the urine. However, *in vitro* assays performed with compounds **19**, **22** and **25** (which all are reasonable stable), showed that especially deterrol (**22**) possesses moderate cytotoxic activity (10 µg/ml inhibits the growth of ECA cells 50 %) and weak mutagenic activity

towards Ames tester strains TA98 and TA100 (2.4 revertants/mg/plate) in the presence of rat liver extract (Anke *et al.*, 1989).

CONCLUSIONS

The secondary metabolism of higher fungi has evolved during millions of years in order to increase the competitiveness of fungi, and produces a large number of biologically active and toxic compounds of which a substantial part is terpenoids. Although the natural function of natural products in general is unknown, many have never the less been suggested to play roles for example as pheromones, as feedants, antifeedants or repellants, as regulators of the development of organisms as well as social behaviours, and in chemical defence systems. All such functions demand of a compound that it possesses biological activities, and the higher the activity is the more efficiently would it normally be able to perform its duties. The likelihood for such compounds to be toxic is therefore not negligible. In view of the fact that mushrooms have a low nutritional value and should be regarded more as a spice than as food in modern cuisine, one way to limit the risks to consumers is simply to avoid consuming excessive amounts of wild mushrooms.

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